The Configuration of Aspartic Acid in Cell Walls of Lactic Acid Bacteria and Factors Affecting the Racemization of Aspartic Acid*

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A micromethod for the determination of the configuration of aspartic acid is described wherein the sample is first treated with aspartic β-decarboxylase to convert L-aspartic acid to alanine. The residual D-aspartic acid and the alanine formed are separated by circular-paper chromatography and estimated with ninhydrin. ε-D-Aspartyl-L-lysine, synthesized by condensing *N*-carbobenzoxy-D-aspartic anhydride with the copper salt of lysine in aqueous pyridine, and by hydrogenolysis, and ε-(aminosuccinoyl)-lysine isolated from cell walls of *Leuconostoc citrovorum* both gave almost exclusively D-aspartic acid upon hydrolysis. ε-L-Aspartyl-L-lysine gave almost exclusively L-aspartic acid under the same conditions, indicating that the aspartic acid in the ε-(aminosuccinoyl)-lysine of *L. citrovorum* must be all of the D form. Application of this analytical procedure showed that some D-aspartic acid is formed during the hydrolysis of proteins or the treatment of L-aspartic acid with hydrochloric acid and that appreciable racemization occurs during hydrolysis of cell-wall material. The extent of such racemization is less in the mucopeptide fraction of cell walls than in the whole wall. It is concluded that at least 90% of the aspartic acid of the intact wall is present in the D-form. The method of drying of hydrolysates and chromatography on Dowex-1 have little or no effect on the configuration of aspartic acid.

The aspartic acid in the cell walls of lactic acid bacteria is predominantly D-aspartic acid (Ikawa and Snell, 1960). However, appreciable amounts of L-aspartic acid were also found and whether this latter form was actually present in the intact walls or arose through racemization of D-aspartic acid was not determined. To study this point further, the extent of racemization of aspartic acid in the absence and presence of cell-wall material, in hydrolysates of proteins, and in cell-wall mucopeptide fractions have been studied together with possible effects of Dowex-1 chromatography and method of drying of hydrolysates. An appreciable fraction of the aspartic acid of cell walls from some lactic acid bacteria appears in hydrolysates as ε-(aminosuccinoyl)-lysine (Ikawa and Snell, 1960). The configuration of the aspartic acid released from this isolated compound and from synthetic ε-(D-aspartyl)-L-lysine by hydrolysis also has been studied.

EXPERIMENTAL

D- and L-Aspartic acids (Asp) were purchased from the California Corp. for Biochemical Research, Los Angeles, Calif.; L-lysyl-L-aspartic acid and *N*-carbobenzoxy-D-aspartic acid were obtained from the Cyclo Chemical Corp., Los Angeles, Calif; and glutamic acid decarboxylase of *Clostridium welchii* was purchased from the Worthington Biochemical Corp., Freehold, N. J. Dr. E. P. Abraham, Oxford, England, generously supplied a sample of ε-(L-aspartyl)-L-lysine (mostly the α-aspartyl isomer but containing some β isomer).

N-Carbobenzoxy-D-aspartic Anhydride.—This was prepared from *N*-carbobenzoxy-D-aspartic acid by the method of John and Young (1954) for the L isomer. Yield, 71%; melting point of recrystallized product, 111–112° (corr); $[\alpha]_D^{25} = +40.7$ (c = 3.3%, w/v, in glacial acetic acid). John and Young (1954) report a melting point of 111° and $[\alpha]_D^{15} = -38.6$ (c = 2.25% in glacial acetic acid) for the L isomer.

Anal. Calcd. for $C_{12}H_{11}O_5N$ (249.2): C, 57.83; H, 4.45; N, 5.62. Found: C, 57.62; H, 4.30; N, 5.74.

ε-(*N-Carbobenzoxy-D-aspartyl*)-L-*lysine.*—This compound was prepared by a modification of the method used by Swallow *et al.* (1958) for the synthesis of ε-(L-aspartyl)-L-lysine. L-Lysine copper complex (2.0 g is equivalent to 8.6 mmoles lysine) was dissolved with warming in a mixture of 40 ml of pyridine and 20 ml of water. The solution was cooled and 1.08 g (4.3 mmoles) of *N*-carbobenzoxy-D-aspartic anhydride were added. After standing overnight at room temperature the mixture was evaporated to dryness under reduced pressure. A 10-ml portion of water was added to the residue and the solution was re-evaporated. This operation was repeated an additional two times to remove most of the pyridine. The residue was then dissolved in 60 ml water, 1 ml of glacial acetic acid was added, and the copper was precipitated by passing it in an excess of H_2S. The mixture was then heated on a steam bath, aerated until the excess H_2S was removed, and filtered. The cupric sulfide precipitate was washed with a small amount of water and the combined filtrates were concentrated *in vacuo* to *ca.* 5 ml. The concentrate was cooled, and the crystalline solid which had separated out during the concentration was filtered off, washed with five 1-ml portions of water, then with ethanol, and dried. Yield, 378 mg (22% based on aspartic acid); mp 219–223° (corr). The melting point of product recrystallized from water was 224–225° (corr).

Anal. Calcd. for $C_{18}H_{25}O_7N_3$ (395.4): C, 54.67; H, 6.37; N, 10.63. Found: C, 54.54; H, 6.48; N, 10.55.

ε-(α-D-*Aspartyl*)-L-*lysine.*—ε-(*N*-Carbobenzoxy-D-aspartyl)-L-lysine (378 mg) was dissolved by warming with 150 ml of water containing 3 ml of glacial acetic acid. The solution was cooled, 1.5 g of 10% palladium on charcoal was added, and hydrogen was bubbled in through a tube fitted with a sintered glass end until the exit gases failed to give a precipitate when allowed to bubble through a solution of barium hydroxide. The catalyst was filtered out and washed with a small volume of water, and the combined filtrates were evaporated to dryness *in vacuo*. The

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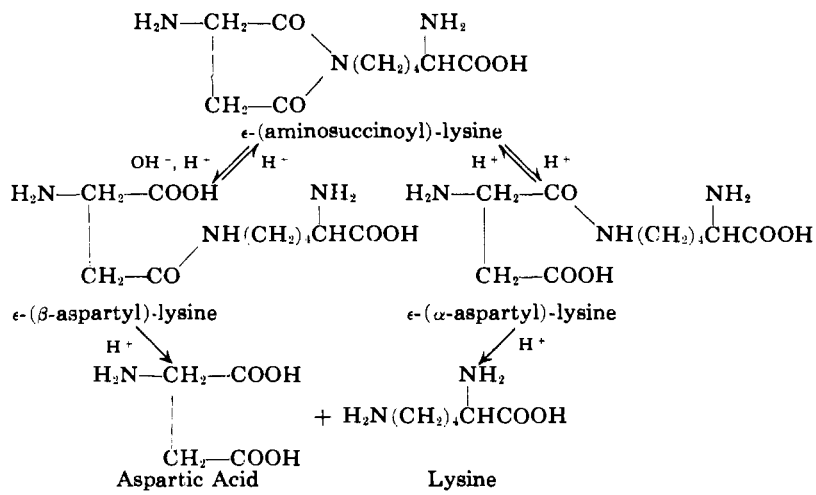
solid residue was recrystallized by dissolving it in a small volume of water with warming and adding two volumes of ethanol. Yield, 182 mg (73%); $[\alpha]_D^{25} = +9.7$ ($c = 1.3\%$ in 0.5 N HCl).

Anal. Calcd. for $C_{10}H_{19}N_3O_5$ (261.3): C, 45.97; H, 7.33; N, 16.08. Found: C, 45.25; H, 7.24; N, 15.70.

Paper electrophoresis in 10% (v/v) acetic acid (Swallow and Abraham, 1958) showed that the compound was essentially all α-isomer. Only traces of the slower-moving β isomer were detected together with traces of aspartic acid and lysine. The α and β isomers migrated at the same rates as the α and β isomers, respectively, of ε-(L-aspartyl)-L-lysine.

Treatment of both ε-(D-aspartyl)-L-lysine and ε-(L-aspartyl)-L-lysine with concentrated hydrochloric acid at 80° for 15 hours (Swallow and Abraham, 1958) led to the formation of the cyclic ε-(aminosuccinoyl)-L-lysine, which, in both cases, behaved identically when chromatographed on paper in pyridine-water, 80:20 (v/v), with a sample of ε-(aminosuccinoyl)-lysine isolated from a hydrolysate of the cell wall of *L. citrovorum* (Ikawa and Snell, 1960).

ε-(Aminosuccinoyl)-L-lysine is readily hydrolyzed by base to give predominantly the β isomer of ε-(aspartyl)-L-lysine (Swallow and Abraham, 1958). When the ε-(aminosuccinoyl)-lysine of *L. citrovorum* cell wall was treated with 0.1 N sodium hydroxide for 1 hour at 25°, the resulting product moved identically with both ε-(D-aspartyl)-L-lysine and ε-(L-aspartyl)-L-lysine on paper chromatograms in 95% ethanol–water–acetic acid, 81:19:1 (v/v/v), a solvent system which does not effect the separation of the α and β isomers. These interconversions are depicted below.



Determination of the Configuration of Aspartic Acid.—An aliquot of solution to be analyzed (containing *ca.* 0.5–1.0 mg aspartic acid) was placed in a small test tube and allowed to evaporate to dryness in a vacuum desiccator containing a beaker of solid sodium hydroxide. To the residue were added 0.1 ml water and 0.2 ml of 1 M acetate buffer (*p*H 5.0) containing in suspension 5 mg/ml of the glutamic decarboxylase preparation from *Clostridium welchii*. Pyridoxal phosphate (0.025 ml, 1 mg/ml) was also added to activate the L-aspartic β-decarboxylase (Meister *et al.*, 1951) present in the enzyme preparation. Control tubes contained 0.1 ml of 1% (w/v) solutions of D- or of L-aspartic acids in place of the 0.1 ml water. The tubes were covered to prevent evaporation and incubated overnight on a shaker at 37°. The contents of the tubes were then centrifuged, and measured aliquots of the supernatant solutions were placed on circular paper chromatograms alongside known amounts of alanine and aspartic acid. The circular chromatograms were developed with 1-butanol–acetic acid–water, 4:1:1 (v/v). The amounts of alanine and aspartic acid present were then estimated by the method of Giri *et al.* (1952).

Results

Effect of Drying Cell-Wall Hydrolysates on Configuration of Aspartic Acid.—When mixtures containing glutamic acid were dried slowly in a vacuum desiccator, γ-glutamyl ester formation with serine occurred to a much greater extent than when the mixtures were dried rapidly, e.g., on a rotary evaporator (Ikawa and Snell, 1961). Control studies in connection with this study showed that the proportion of D-aspartic acid present in cell-wall hydrolysates was the same whichever of the two drying procedures was used.

Effect of Acid Treatment on the Configuration of Aspartic Acid.—D-Aspartic acid was heated for various lengths of time in 3 N and 6 N hydrochloric acid (Table I). The usual conditions of hydrolysis employed in hydrolyzing cell walls, i.e., 3 N hydrochloric acid for 18 hours at 100–105°, resulted in approximately a 5% conversion of D-aspartic acid to L-aspartic acid. Heating in 6 N hydrochloric acid for longer periods of time resulted in more extensive racemization and after 8 days the aspartic acid was almost completely racemized. D-Asparagine behaved similarly to D-aspartic acid. The addition of a large excess of starch did not materially affect the extent of racemization of aspartic acid.

The Configuration of the Aspartic Acid in the ε-(Aminosuccinoyl)-lysine from the Cell-Wall Hydrolysate of L. Citrovorum.—Since the peptide bond in ε-(aspartyl)-L-lysine and in ε-(aminosuccinoyl)-L-lysine, the product formed from the former compound under acidic conditions, is extremely stable to acid (Swallow and Abraham, 1958), samples were hydrolyzed in 6 N hydrochloric acid for 42 hours. The results (Table I) show that ε-(D-aspartyl)-L-lysine gave principally D-aspartic acid and ε-(L-aspartyl)-L-lysine gave principally L-aspartic acid. The predominance of D-aspartic acid resulting from the hydrolysis of ε-(aminosuccinoyl)-lysine from *L. citrovorum* cell wall shows that the configuration of the aspartic acid moiety must be D. Hydrolysis of L-lysyl-L-aspartic acid also gave principally L-aspartic acid, although the extent of racemization was greater than that observed for aspartic acid alone under the same conditions.

Effect of Dowex-1-Column Chromatography on the

TABLE I

CONFIGURATION OF THE ASPARTIC ACID RESULTING FROM THE HYDROCHLORIC ACID TREATMENT OF ASPARTIC ACID AND SOME OF ITS DERIVATIVES[a]

Substance	HCl Concn (N)	Hours at 100–105°	D-Asp / Total Asp × 100
D-Aspartic acid (control)	0	0	96–100
L-Aspartic acid (control)	0	0	0–5
D-Aspartic acid (0.5%)	3	18	94
D-Aspartic acid (0.5%) + starch (10%)	3	18	94
D-Aspartic acid (0.5%)	6	42	89
D-Aspartic acid (0.5%) + starch (10%)	6	42	86
D-Aspartic acid (0.5%)	6	192	62
D-Asparagine monohydrate (0.5%)	6	6	94
D-Asparagine monohydrate (0.5%)	6	42	86
ε-(α-D-Aspartyl)-L-lysine (0.25%)	6	42	88
ε-(Aminosuccinoyl)-lysine from *L. citrovorum* cell wall	6	42	91
ε-(L-Aspartyl)-L-lysine (0.25%)	6	42	17
L-Lysyl-L-aspartic acid (0.25%)	3	18	23

[a] Aspartic acid and its derivatives were treated as indicated. The figures in parentheses give the concentrations (w/v) of the substance. The resulting mixtures were evaporated to dryness in a vacuum desiccator containing solid sodium hydroxide, and the configuration of the aspartic acid was determined on the residue.

Configuration of Aspartic Acid.—Since the method used here to determine configuration of aspartic acid necessitates the prior removal of interfering substances, especially alanine, hydrolysates of the more complex substances or mixtures were chromatographed on a Dowex-1 column in order to isolate the aspartic acid fraction. To determine whether this chromatographic step led to any racemization, 5-mg samples of D-aspartic acid in 0.5 ml water were chromatographed on a 10 × 400 mm column of 200–400 mesh Dowex-1 (acetate) and eluted by developing with 0.5 M acetic acid (Hirs *et al.*, 1954). The aspartic acid isolated from the effluent was determined to be 92 and 95% D-aspartic acid in two separate experiments. Since untreated D-aspartic acid assays 96–100% D-aspartic acid (Table I), only about 5% racemization, at most, results from the Dowex-1 chromatography of D-aspartic acid.

Configuration of Asvartic Acid in Protein and Cell-Wall Mucopeptide Hydrolysates.—Casein was hydrolyzed at several concentrations of acid and varying temperatures and the configuration of the resulting aspartic acid was determined (Table II). From 8.5–14% of the total aspartic acid was D-aspartic acid, indicating that some racemization occurred during hydrolysis. Ovalbumin and ribonuclease on hydrolysis also yielded some D-aspartic acid. The amount formed (7% of the total aspartic acid) appears to be slightly less than was the case with casein.

The proportions of D- and L-aspartic acids in hydrolysates of cell walls had been reported previously (Ikawa and Snell, 1960). Hydrolysis of the mucopeptide fraction of the cell walls of *Streptococcus faecalis*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus acidophilus*, and *Leuconostoc mesenterosides* gave a substantially higher proportion of D-aspartic acid (Table II) than did the whole cell wall, and the high values

TABLE II

CONFIGURATION OF ASPARTIC ACID IN HYDROLYSATES OF PROTEINS AND CELL-WALL MUCOPEPTIDE. EFFECT OF HEATING OF ASPARTIC ACID WITH CELL WALL[a]

Substance Hydrolyzed	HCl Concn (N)	Hours at 100–105°	D-Asp / Total Asp × 100
Casein (1%)	1	42	14
Casein (1%)	3	18	8.5
Casein (1%)	6	18	9
Casein (1%)	3	18 (120°)	13.5
Casein (1%)	3	18 (80°)	13.5
Casein (1%)	6	18 (80°)	10.5
Ovalbumin, cryst. (1%)	3	18	7
Ribonuclease, cryst. (1%)	3	18	7
Mucopeptide[b] of *S. faecalis* cell wall (1%)	3	18	91
Mucopeptide of *L. casei* cell wall (1%)	3	18	72, 84
Mucopeptide of *L. helveticus* cell wall (1%)	3	18	57
Mucopeptide of *L. acidophilus* cell wall (1%)	3	18	82
Mucopeptide of *L. mesenteroides* cell wall (1%)	3	18	74
D-Asp (0.1%) + *L. plantarum* cell wall (1%)	3	18	76
D-Asp (0.1%) + *L. pentosus* cell wall (1%)	3	18	72
D-Asp (0.1%) + *L. plantarum* teichoic acid[c] (1%)	3	18	74
L-Asp (0.1%) + *L. plantarum* whole cells (1%)	3	18	6.5
L. plantarum whole cells, lyophilized (1%)	3	18	7

[a] Samples were treated as indicated. Numbers in parentheses give the concentrations (w/v) of the reactants. Hydrolysates, after drying, were chromatographed on Dowex-1 (acetate) (10 × 400 mm, 200–400 mesh) with 0.5 M acetic acid development (Hirs *et al.*, 1954). The aspartic acid fraction was concentrated to dryness on a rotary evaporator. The residue was dissolved in 2.5–5.0 ml water and aliquots were analyzed for aspartic acid configuration. [b] The hot trichloroacetic acid–insoluble, sodium hydroxide–insoluble fraction of cell wall (Ikawa, 1961). [c] The nondialyzable hot trichloroacetic acid–soluble fraction of *L. plantarum* cell wall (Ikawa, 1961).

found for *S. faecalis*, *L. casei*, and *L. acidophilus*, when compared with the 8.5–14% of D-aspartic acid found in the aspartic acid fraction of casein hydrolysates, indicate that the aspartic acid in the cell walls of these organisms is at least 90% of the D-form. Although the situation of the aspartic acid in *L. mesenteroides* cell wall is probably similar, the situation in *L. helveticus* cell wall still remains uncertain. Isolation of the mucopeptide fraction of the cell walls of these organisms involved treatment with 0.1 N sodium hydroxide for $^1/_2$–1 hour at room temperature (Ikawa, 1961). It is possible that a slight amount of racemization of aspartic acid may have taken place during alkali treatment to decrease the yield of D-aspartic acid from the mucopeptide fraction.

Effect of Hydrolysis in the Presence of Cell Walls on the Configuration of Aspartic Acid.—When D-aspartic acid was added to samples of cell walls of *Lactobacillus plantarum* and *Lactobacillus pentosus*, and the mixtures were subjected to hydrolysis, a considerable amount of racemization was observed (Table II). The extent of racemization is much greater than can be accounted for on the assumption that the L-aspartic acid arose from the cell wall, since cell-wall preparations of these organisms digested with proteolytic enzymes contain

less than 1% of total aspartic acid (Ikawa and Snell, 1960). Extensive racemization was also observed on heating D-aspartic acid with the teichoic acid fraction of *L. plantarum* cell wall (Table II). In this case contamination by L-aspartic acid is excluded since D-alanine is the only amino acid known to occur in this fraction (Armstrong *et al.*, 1958; Ikawa, 1961). These results therefore indicate that the teichoic acid fraction of cell walls must contribute to the racemization of aspartic acid during hydrolysis. This was also indicated by results cited previously which showed a higher proportion of D-aspartic acid in mucopeptide hydrolysates than in the corresponding cell-wall hydrolysates. However, when cell-wall material is sufficiently diluted by other cell constituents this increased racemization is not observed, as indicated by the fact that aspartic acid isolated from hydrolysates of whole cells of *L. plantarum* contained only 7% D-aspartic acid (Table II). When cells of *L. plantarum* were hydrolyzed in the presence of added L-aspartic acid, the resulting aspartic acid again contained only 6.5% of the D form. These latter results are in agreement with those of Camien (1951), who found that the aspartic acid of *L. arabinosus* contained about 5% of the D form.

Discussion

It is generally assumed, for good reasons, that proteins are composed entirely of L-amino acids and that any D forms observed have arisen through racemization during hydrolysis. Chibnall *et al.* (1940) did a quantitative copper salt isolation of the aspartic acid resulting from the hydrolysis of ox heart protein in 20% hydrochloric acid for 22 hours, and concluded from optical rotation data that their product contained *ca.* 5% of the D isomer. Camien (1951), using a differential microbiological assay system which determined L- and total aspartic acids, estimated 1–5% of the aspartic acid in casein hydrolyzed in 8 N hydrochloric acid for 20 hours could be D-aspartic acid. The results of the present study, using a different method to determine configuration, indicate *ca.* 7% of the D isomer in the aspartic acid of ovalbumin and ribonuclease hydrolysates, and slightly higher values (8.5–14%) for casein hydrolysates. Some tendency therefore exists for aspartic acid to racemize during protein hydrolysis. A similar tendency also exists when pure aspartic acid is heated with acid, and this becomes very appreciable on prolonged treatment. However in this connection Levene and Steiger (1930) were unable by optical rotation measurements to detect any racemization when glycyl-L-asparagine or glycyl-L-asparagine anhydride were heated at 100° with 20.3% hydrochloric acid for 36 hours. Racemization from other factors such as method of drying of hydrolysates and Dowex-1 chromatography seems to be slight at most.

From these and other data, one might reasonably expect to find about 10% (5–15%) of racemized aspartic acid on hydrolysis of samples containing a single isomer, a conclusion that suggests that the aspartic acid in the cell walls of lactic acid bacteria is at least 90% of the D form. The supporting evidence is: (a) the D configuration of the aspartic acid in the ε-(aminosuccinoyl)-lysine isolated from hydrolysates of *L. citrovorum* cell walls, (b) the higher proportion of D-aspartic acid in hydrolysates of the cell-wall mucopeptide fraction than in hydrolysates of the corresponding whole cell walls, and (c) the apparent racemization-promoting tendency of cell-wall fractions. This conclusion is in agreement with that of Toennies *et al.* (1959) for *S. faecalis* 9790, in which 91% of the aspartic acid in the cell wall was D. Since the aspartic acid in the ε-(aminosuccinoyl)-lysine from *L. citrovorum* cell wall represents only about 1/4 of the total aspartic acid of the cell wall, the conclusion that the configuration of the aspartic acid in the cell wall is the same as that in this stable peptide is true only if the aspartic acid in the peptide is representative of the aspartic acid in the cell wall. That this is the case is supported by the finding of approximately equimolar amounts of lysine and aspartic acid in the cell walls of a number of organisms (Ikawa and Snell, 1960). The occurrence of equimolar amounts of lysine and aspartic acid in cell walls would be expected if these two amino acids can be replaced by the neutral α,α′-diaminopimelic acid in the cell walls of some organisms.

The stable cyclic peptide ε-(aminosuccinoyl)-lysine was first shown to result from the hydrolysis of bacitracin (Lockhart and Abraham, 1956) and has been found to be a common constituent in hydrolysates of cell walls (Cummins and Harris, 1956; Swallow and Abraham, 1958; Ikawa and Snell, 1960). Both ε-(α-L-aspartyl)-L-lysine and ε-(β-L-aspartyl)-L-lysine were synthesized by Swallow *et al.* (1958) and were shown on acid treatment to yield a cyclic product that could not be distinguished from the ε-(aminosuccinoyl)-lysine from bacitracin or *L. brevis* cell-wall hydrolysates; the only uncertainty was in the optical configurations of the amino acids in the natural products. The present experiments show that ε-(α-D-aspartyl)-L-lysine cannot be distingiushed chromatographically from ε-(α-L-aspartyl)-L-lysine, and that their cyclized products also are indistinguishable chromatographically. Our previous results, however, showed the configuration of lysine in the cell wall to be L-lysine (Ikawa and Snell, 1960), while results presented herein show that the cyclic peptide isolated from hydrolysates of the cell walls of *L. citrovorum* is derived from ε-(D-aspartyl)-L-lysine.

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